THE USE OF THE COLORIMETER IN THE INDICATOR METHOD OF H ION DETERMINATION WITH BIOLOGICAL FLUIDS

B. M. DUGGAR
Physiologist to the Missouri Botanical Garden, in Charge of Graduate Laboratory
Professor of Plant Physiology in the Henry Shaw School of Botany of Washington University

AND C. W. DODGE
Formerly Rufus J. Lackland Fellow in the Henry Shaw School of Botany of Washington University

In recent years it has become essential that physiologists, bacteriologists, and biochemists generally shall be able to determine accurately and conveniently the approximate actual reaction or hydrogen ion concentration of solutions or media of various types. It is almost inconceivable that any extensive work with biological solutions, including fermentation products and culture fluids, may proceed without adequate consideration of this factor.

It is clearly recognized that as an absolute standard in the measurement of the H ion concentration of solutions one must rely upon the use of the hydrogen electrode, whether with or without the more recent developments in the way of direct-reading potentiometers. Nevertheless, the electrical or gas-chain method requires considerable physico-chemical experience and a type of apparatus not commonly available to the physiologist or bacteriologist.

To students working in the fields just mentioned and employing nutrient solutions, decoctions, plant juices, the products of fermentation, etc., the indicator method in its present standard of development makes a strong appeal. This is true because: (1) an adequate degree of accuracy is usually attainable by this means, especially if the standard solutions are occasionally checked by the electrometric method; (2) the indicator method has special application where the quantity of material available may be small and the determinations need to be made promptly, as occasions

arise; and (3) it requires no thermal bath or other supplementary apparatus except perhaps a colorimeter in the cases to be discussed later. So long as the test solutions or media employed are colorless, or practically so, the indicator method presents now no difficulties which are not readily precluded by a little experience.

Rapid advances, however, have been made during the past few years in the perfection of buffered or standard solutions of carefully determined hydrogen ion concentration with which to compare the fluids studied. The contribution made by Clark and Lubs ('17) in respect to standard solutions is of almost equal importance to the excellent choice of indicators presented by them. In view of the availability of the work of Clark and Lubs and the detailed discussion by them it is unnecessary to refer to the preparation of such standard solutions further than to emphasize the necessity for all the refinements prescribed. In the work here reported, as well as in other studies now in progress, use has also been made of the standard solutions of Sörensen ('09–'10).[1] We have found, however, that the citrate and glycocoll mixtures undergo rapid deterioration, while the thallate, phosphate, and borate mixtures are much more stable. All solutions, whether the prepared standards or the stock solutions from which these are made, should be kept in well-seasoned glassware, and, so far as possible, the same container should be employed for a particular ionic concentration. Moreover, since the introduction of the thymol, cresol, phenol, and certain benzene products, it is no longer necessary to choose a doubtful indicator from the extensive charts of the earlier investigators, such as those of Salm ('06).

The newer indicators exhibit, for the most part, brilliant color changes throughout the range of P_H values usually required; although, as subsequently emphasized, particular care is required in the case of colored test fluids both in respect to the choice of the indicator and in checking the ac-

[1] Reference is here made to Sörensen's paper in the Carlsberg Compt. rend. des Trav. rather than to the other source of this material—Biochem. Zeitschr., 1913—in view of the fact that in the former only is a correction made (at the end of the paper) for an error in stating the amount of the phosphate employed.

curacy of the determinations made near its limits of brilliancy by another indicator with slightly overlapping color change. Moreover, it is often desired to use a particular indicator at or near the limits of its usual range. Thus methyl red, extremely serviceable between P_H 4.4 and P_H 6.0, just fails to completely cover the range of certain nutrient solutions and plant juices frequently employed in the culture of fungi. The limitation, however, is really in the ability of the unaided eye to detect readily the slight differences when a certain redness (or yellowness) is approached. The difficulty of color in the medium under investigation, however, is the most serious. In our work the indicator solutions have been prepared by using the quantities recommended by Clark and Lubs in 50 per cent ethyl alcohol. These are preserved in amber dropping bottles.

When a careful technique is established the degree of accuracy sufficient for all practical purposes is assured in the examination of colorless solutions by the following procedure: Small test-tubes containing a measured quantity (usually 5 or 10 cc.) of the standard solutions are arranged in open racks provided with a white paper background. A series is prepared for each indicator employed, and the P_H values may differ by .1 or .2, depending upon the accuracy required. A definite and constant quantity of the indicator, usually 2 or 3 drops, is placed in each tube. The same quantity of the sample or test solution is placed in a similar test-tube and the indicator added as before. The samples are then compared with the various standards in a uniform light and an exact match is obtained. A characteristic of most fluids or media with which the physiologist deals is color, and this has operated in the past more or less to interfere with the correct determinations by the indicator method.

Early investigators were disturbed by the presence of color in the solutions studied, and various methods were employed to counteract this source of error. Sörensen ('09–'10) proposed a method of dealing with colored solutions, whereby the natural test solution was matched in color by means of neutral dyes used in the standard solutions, before the addition of

the indicators. Aside from being tedious, this method gave at best only a rough comparison, exhibiting obvious errors and leaving much to the personal equation or opinion of the observer.

Walpole ('10, '10[a]) introduced a logical procedure involving the use of the colored test solution as a shield to compensate for the color of the sample under observation. He arranged a simple device which when employed for H ion determination consisted of a blackened frame or support holding four glass cells in two similar columns. Each column consists of a cell surmounted by a Nesslerizing tube, and each column is illuminated from a dull white surface below, reflecting the light upward. In the one column the lower tube contains the colored test fluid or sample plus indicator, and the upper tube water; while in the other column the upper tube contains the standard solution (in that case Sörensen's), and the lower the test fluid as shield. In each column the light passes through the colored sample and through a colorless solution, either one or the other, but not both, containing the indicator. The contents of the tube with standard solution may be changed, or other cells introduced differing slightly in H ion concentration, until, on looking down through the column, an exact match is obtained. Although obviously defective optically, this simple tintometer is serviceable. The apparatus has also been used considerably for titration work.

Independently, Hurwitz, Meyer, and Ostenberg ('16) devised at about the same time another simple apparatus for the compensation of color when the indicator method of H ion determination is employed. In this, designated a comparator, the same principle as above is applied, but the stand is so formed that four test-tubes are supported vertically in pairs and in the same horizontal plane. The system thus consists (1) of one pair of tubes (in the direction of the line of vision) with the nearer tube containing the standard solution and indicator, in front of a "shield" tube containing the colored sample; while (2) the other pair of tubes consists of one tube containing the sample and indicator, shielded beyond by a tube of water or of standard solution without

indicator. The disadvantages of this instrument are practically the same as those mentioned above, but it has obvious advantages over the usual test-tube comparison.

In some studies on the nutrition of the fungi wherein a variety of plant decoctions was employed the writers experienced the usual difficulties in rapidly and accurately employing the indicator method for determining the active acidity of these media. The plant decoctions were made in accordance with our usual method, which consists in slicing the product, or cutting it into short lengths, adding the requisite amount of water, and autoclaving at 10–15 pounds pressure for one hour. The effect of this autoclaving for extraction, together with another interval of 15–30 minutes for sterilization, after filtering into flasks, is to yield a decoction which is often highly colored. Solutions prepared from rhubarb, celery, carrots, prunes, apples, mangolds, and sweet potatoes gave, as might be expected, more pronounced color than those made from sugar beets, potatoes, and green beans. In any case, after repeated sterilizations the deepened color became a source of considerable annoyance. It should be stated that this work was begun prior to 1917, so that we were not at first in possession of the newer indicators.

In any case it seemed wise to investigate the possibility of employing the colorimeter in such work. At first no reference could be found in the literature to the use of the colorimeter in that way. Nevertheless, Veley ('06), Tizard ('10), Walpole, and perhaps others had apparently, with no great amount of consistency, employed the colorimeter in the determination of the constants of indicators and in other related work. Prideaux ('17) expresses regret that all necessary conditions—referring especially to the concentration of the indicators and to whether or not a colorimeter was used —have not been carefully specified for each indicator constant, so that it might be employed in the colorimetric determination of the H ion with greater confidence. It would appear, however, that he has employed the colorimeter directly in the determination of H ion concentration, because of the following statement: "The accuracy of a colour com-

parison by eye cannot easily be brought within 0.1 in the hydrogen exponent. With a colorimeter it is perhaps possible to obtain results agreeing to 0.01, but such an accuracy is unnecessary and is not practicable in ordinary tests of acidity or titrations.'' Our attention, however, was not directed to this fact until after the completion of our method, and indeed only after a careful search of his book with the idea of determining whether such references could be found in related literature.

In this work a complete Kober ('17) nephelometer-colorimeter was employed, as this instrument happened to be at hand. In reality, it possesses two distinct advantages, namely, uniform and effective source of light, and protection from side illumination. It was realized that since the colorimeter was only required in the study of colored solutions, the important factor in this case was to apply effectively the method of shield solutions. This was ultimately accomplished so satisfactorily that the defects of the comparator method were entirely obviated, while all the advantages of the colorimeter were retained.

The method consisted simply in arranging for each side of the colorimeter a pair of cups slipping to a certain depth (noted later) one into the other, as shown in fig. 1. The method of procedure is then as follows: For the left-hand set, or column, water (or colorless standard solution) is used in the outer cup, and the colored test fluid plus indicator in the inner cup. After adjustment, this set is not removed from the colorimeter during an observation. In the case of the right-hand set the outer cup contains the colored test fluid, while the inner cup is for the standard solution plus indicator. This set is placed on the right for convenience, as it may be necessary to compare with the test fluid a series of standards until an exact match is obtained. A rough comparison is of course made before selecting the standard solution for comparison. In each case the column must contain an equal depth of colored test solution and of standard or colorless liquid, the indicator being in the standard in the one case and in the test solution in the other. There are no

optical difficulties, and unless the indicator combines with the test solution, the comparison may be perfect.

In order that equal depths of liquid may be examined it is only necessary to know, or gauge by suitable washers, the distance *a* to *b* in the figure; then (if the inner cup is not the exact length of the plunger) after placing the cups on the carriers they are raised until the tip of the plunger barely touches the bottom of the inner cup when the position is read on the scale. The cups are then lowered to a distance equal to the line *ab*. It is to be noted that the quantity of solution to be placed in the cups is not necessarily determinate, so long as there is at least sufficient depth in each to equal the distance *ab*. We have found that a depth of 10–15 mm. of liquid is not too great with the instrument employed, assuming that the red indicators are utilized. In order that air bubbles may not catch under the lens the inner diameter of the inner cup should be 4–5 mm. greater than the diameter of the plunger, and similarly the inner diameter of the outer cup should be correspondingly greater than the outer diameter of the inner cup. It is also evident that good optical glass should be employed for the bottoms of the cups.

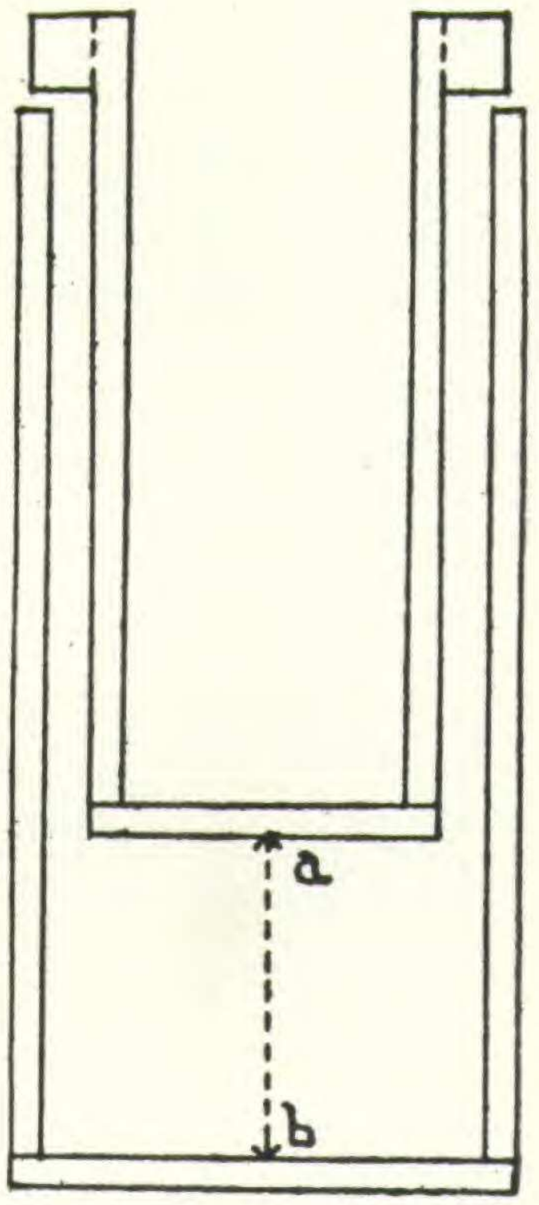

Fig. 1. Special colorimeter cups.

It is believed that the colorimeter may be employed in this work almost as rapidly as the comparator, and certainly with greater confidence and accuracy. For rapid work it is essential that one should understand the particular indicator in colored solution; likewise the effects of the quality of light employed on the color of the field, but these are minor difficulties. The red indicators, with color change red-yellow or yellow-red, have proved particularly satisfactory. In the use of these it soon became obvious that the usual P_H range of each might be considerably extended by the use of the colorimeter, but in any case the extent of the useful range is somewhat

dependent upon the intensity of color in the test fluids. With a weak beet decoction plus acid or phosphates methyl red was useful from P_H 3 to P_H 6.8, and phenol red (phenol-sulphonphthalein) from P_H 6.4 to P_H 9.0.

Any refinements in the use of the simple indicator method of hydrogen ion determination should find many applications in the wide range of plant physiological studies with both lower and higher organisms. The importance of this factor of active acidity has been repeatedly urged in recent work, yet it is not receiving general consideration. In medicine the value of such determinations has gradually become apparent following the interesting development of views regarding neutrality regulation in animal fluids (compare some of the work of Henderson, '08, '09, '09[a], of Henderson and his associates, and others).

In making determinations of the hydrogen ion concentration of the blood, Levy, Rowntree, and Marriott ('15) have employed a dialysis method, used also in a study of the buffer value (Levy and Rowntree, '16) of this fluid, while a more accurate modification of the method (Marriott, '16) is used to determine the alkali reserve of the blood plasma. In plant studies the matter of neutrality regulation might seem on first thought to be of relatively little consequence, because of the diversity of reaction. The extent of the acid reserve in a general way is appreciated, but the determination of this has been largely incidental to other considerations. It would be interesting to know to what extent an acid reserve is a general characteristic of plant metabolism.

From the studies reported on animals it would appear that the protoplasm of many organisms is approximately neutral, but the indications would seem to be that plant protoplasm is often far from neutral, frequently exhibiting a relatively high acidity. It is still a question, however, to what extent the P_H determined for the juice (as a whole), representing to a large extent the contents of the vacuoles, is an index of the reaction of the protoplasm (Haas, '16). The case of certain citrus fruits is, of course, an exception, since here the more acid juice is contained in special sacs. Aside from this

instance there is still exhibited a remarkable $P_{H'}$ range, determined by Haas for the juices of certain higher plants to be between P_H 3.0 and P_H 7 or 8. This, moreover, is more or less comparable to the relation of certain mould fungi (notably *Penicillium italicum* and *Aspergillus niger*) to the reaction of nutrient media.

From the work here reported on the use of the colorimeter it may be concluded that (1) the difficulties involved in the approximate determination of the hydrogen ion concentration of solutions exhibiting color may be largely overcome; and (2) the useful range of certain brilliant indicators may be so considerably extended that the number of indicators employed may be materially reduced.

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